Effects of ethidium bromide on the production of ribosomal RNA in cultured mouse cells

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ABSTRACT
A treatment of primary mouse kidney cell cultures with 5 µM or 10 µM Ethidium Bromide (Eth Br) reduces the transcription of nuclear-coded genes and especially of ribosomal RNA genes. This effect was consistently observed when comparing drug-treated and control cells for (i), the incorporation of $^3$H uridine into total nuclear and Hn RNAs, and (ii), the activity levels of Form A and B RNA polymerases as determined in isolated nuclei. It became more pronounced with exposure time; however, after removal of the drug, there was a progressive recovery of RNA synthesis culminating in the complete reversal of the drug effect. That this effect is probably not due only to the suppression of mitochondrial protein synthesis by the drug, is shown by a comparative study of the effects of chloramphenicol treatment. In addition, in the cytoplasm Eth Br depresses the labeling of 28 S rRNA more than that of 18 S whereas no abnormal accumulation of 28 S rRNA is observed in the nucleus. It is suggested that Eth Br may affect either the stability of the 28 S rRNA or its rate of formation from the 32 S precursor.

INTRODUCTION
A large number of effects of ethidium bromide (Eth Br, a phenanthridine dye) in living organisms have been reported. The drug (Eth Br) intercalates between adjacent base pairs of double stranded regions of nucleic acids (1). This intercalation causes alteration of the double helix reflected in local extension and unwinding of the duplex structure. Eth Br has been used as probe for the structure and properties of closed circular DNA (2, 3), transfer RNA (4), SV40 transcriptional complexes (5), ribosomes (6) nucleosomes (7) and chromatin (8, 9).

The biological consequences of the intercalating properties of Eth Br are multiple. At very low concentrations ($\leq 1$ µg/ml i.e. 2.5 µM) the drug is known to preferentially inhibit the synthesis of mitochondrial DNA (10, 11), RNA (12, 13, 14) and proteins (15, 16, 17). At high concentrations (500-570 µM), the drug inhibits the processing of 45 S nuclear RNA to other species in the ribosomal RNA maturation sequence in L 1210...
mouse lymphoma (18) and stabilizes the heterogeneous nuclear RNA of KB cells (19).

Little, however, is known concerning the effects of relatively low Eth Br concentrations on the synthesis of RNA species of nuclear origin in cell cultures.

The present study shows that treatment of mouse cell cultures with 5 μM or 10 μM Eth Br reduces the transcription of nuclear-coded genes and especially of ribosomal RNA genes. Although this inhibition becomes more pronounced with exposure time, it is probably not simply due to the suppression of mitochondrial protein synthesis by the drug. In addition, Eth Br treatment appears to affect either the stability of the 28 S rRNA or its rate of formation from the 32 S precursor.

MATERIALS AND METHODS

Culture conditions, labeling and drug-treatment

Primary baby mouse kidney (BMK) cell cultures (20) were prepared from 10 day-old Swiss albino mice and grown in 10-cm plastic petri dishes under conditions described earlier (21). Confluent cultures contained approximately 6 x 10⁴ cells/cm². One day after they reached confluence (or, where specified, at the time when they were 50 % confluent) the cultures were exposed either to Eth Br (2 or 4 μg/ml) or to chloramphenicol (50 μg/ml) for 6 hr and labeled with 10 μCi/ml ³H-uridine (20-25 Ci/m mole, CEA, Saclay, France) in the presence of 5 μg/ml cold uridine (Calbiochem, A grade) for the last 3 hr of the drug treatment. Eth Br was supplied by Calbiochem, chloramphenicol by Roussel UCLAF. In some experiments, the cultures were infected with SV40 one day after they have reached confluence, at a multiplicity of about 50 PFU/cell. After a 1-hour adsorption at 37°C, cells were refed with Dulbecco's medium without added serum. Cells were exposed to Eth Br (2 or 4 μg/ml) from 3 to 9 hours after infection and labeled with ³H-uridine under the same conditions as described above for the uninfected cultures. In one experiment, the SV40-infected cells were exposed to Eth Br (2 μg/ml) from 2.5 to 9 hr after infection, and were labeled with ³H-uridine from 3 hr to 9 hr p.i. The SV40 transformed mouse cell clonal subline, SVMK clone 9, originated from a line established by SV40 infection of primary mouse kidney cell cultures (22). The established (monkey kidney) cell line CV-1, and the transformed cell lines SVMK clone 9 were propagated in Eagle's medium which was supplemented with 10 % calf serum (Gibco). CV-1 or SVMK clone 9 cells were seeded in 10-cm plastic
petri dishes at $5 \times 10^6$ cells per dish in the maintenance medium. They were exposed for 6 hr to Eth Br (2 or 4 µg/ml) at 15 hr after plating and were labeled during the last three hours of the Eth Br treatment. Primary cultures of monkey (papio papio) kidney cells were prepared in a same way as the primary cultures of mouse kidney cells.

In all experiments, the cells used to examine the effects of Eth Br or chloramphenicol were exposed at the appropriate time to fresh medium containing the drug and without serum. Thus the BMK cells which were 50% confluent (cell density: $3 \times 10^6$ cells/cm$^2$) at the time of initiation of treatment were maintained at this cell density. Exposure to the media containing $^3$H-uridine was conducted in the presence of the same concentration of drug and in the absence of serum. In some experiments cells were treated with 0.05 µg/ml actinomycin D to block ribosomal RNA synthesis (23). In these experiments, actinomycin D (Sigma) was kept in an aqueous solution at 0.2 µg/ml and appropriate volumes added to the cultures 30 minutes before the $^3$H-uridine.

In all experiments, cultures treated with Eth Br or chloramphenicol were compared with controls not treated with the drug.

**Cell fractionation, RNA isolation, fractionation of cytoplasmic RNA**

At the end of the labeling period, cells of two dishes were washed twice with 5 ml per dish of ice-cold isotonic buffer I (10 mM triethanolamine pH 8, 25 mM NaCl, 5 mM MgCl$_2$, 0.25 M sucrose). After draining, 0.5 ml per dish of cold buffer I containing 1% Nonidet P 40 (NP 40, Shell Chemical Co) was added for 5 minutes. The lysate was scraped and homogenized with 3 strokes of a tight pestle in a Dounce tissue homogenizer. Nuclear and cytoplasmic fractions were separated by centrifugation at 2,500 rpm for 5 minutes at 4°C, and recovered in the pellet and the supernatant respectively. The RNA from the cytoplasmic fraction was extracted by the phenol-chloroform method according to Rosenthal (24). The nuclear pellet was washed by suspension in phosphate-buffered saline and centrifugation. Nuclear RNA was extracted by the method of Scherrer (25), including a DNase treatment. Cytoplasmic RNA was fractionated by oligo d(T)-cellulose chromatography (24) in order to obtain poly(A)$^+$ RNA and poly(A)$^-$ RNA.

**Sedimentation in sucrose gradients.**

RNA samples in 100 µl buffer were layered on top of a (4.0 ml) linear (15-30% w/v) sucrose density gradient made in 10 mM triethanolamine, pH 7.4, 50 mM NaCl, 1 mM EDTA and were centrifuged in a Spinco SW 56 rotor at 20°C and 54,000 rpm for 110 min. 4-drop fractions were collected from the
bottom of the tubes and dried on filter-paper discs which were then washed with 5% trichloroacetic acid at 4°C and counted.

**Polyacrylamide gel electrophoresis.**

Electrophoresis of RNA in polyacrylamide gel of uniform concentration (2.2%) was carried out as described by Mirault and Scherrer (26) except that our E-RNA buffer had the following composition: 0.02 M citric acid, 0.002 M EDTA·H₂O, neutralized at pH 7.4 by triethanolamine (K. Scherrer personal communication). Details are given in legend to Figure 2.

**RNA and DNA assays.**

DNA and RNA from the nuclear fraction and RNA from the cytoplasmic fraction were extracted according the Schmidt and Thannhauser's procedure (27) and the nuclear DNA content was determined by the diphenylamine method with 2 deoxy-D-ribose (crystalline, Sigma grade). A 100 μl aliquot of the alkaline hydrolysate containing the nucleotides derived from the nuclear or cytoplasmic RNA was mixed in 2 ml of ethanol and this solution was put in 10 ml scintillation fluid in order to determine radioactivity. The incorporation of ³H-uridine into either nuclear or cytoplasmic RNA was expressed as cpm/μg of DNA. RNA concentration from the cytoplasmic RNA preparations was determined by absorbance measurement at 260 nm, assuming a specific absorbance of 0.024 cm²/μg.

**In vitro assay of RNA synthesis.**

Two parallel sets of drug-treated and control mouse kidney cells were lysed with buffer I containing 1% NP-40 as described above. The lysates were then homogenized with 10 strokes of a tight pestle in a Dounce tissue homogenizer. The homogenates were centrifuged at 2,500 rpm for 5 minutes at 4°C. The nuclear pellets were washed by suspension in phosphate-buffered saline, pelleted again, drained and gently resuspended in Tris 50 mM, HCl 50 mM, mercaptoethanol 2 mM. The assays of RNA synthesis were essentially performed as described by Cox (28).

The reaction mixtures had a final volume of 0.2 ml and contained 250 mM (NH₄)₂SO₄, 60 mM Tris-HCl pH 7.9, 0.6 mM EDTA, 2.4 mM MnCl₂, 6 mM dithiothreitol, 250 μg Heparin (Choay), 0.6 mM each of GTP, CTP and ATP, 45 μM UTP (Boehringer, Mannheim) and 5 μM ³H-UTP (11 Ci/mmole, Radiochemical Center, Amersham). Assays were carried out in the presence and absence of 1 μg/ml -aminitin in reaction mixture. The reaction mixtures were incubated at 21°C for 1 hr and the reaction terminated by adding 3 ml of 5% trichloroacetic acid at 4°C. After 30 minutes on ice, the precipitates were collected on Whatman GF/C filters, washed with 100 ml 2.5% trichloroace-
tic acid, rinsed with 5 ml 95 % ethanol, dried and counted. Background counts were determined by adding 5 % trichloroacetic acid immediately after addition of the samples to the reaction mixtures and were subtracted from the counts measured after incubation at 21°C. Enzyme activities were calculated in $^3$H UMP incorporated per unit of DNA. Under the experimental conditions used (high salt concentration) form C activity was very low (28) form B activity was calculated as the difference between the total activity (without α-aminitin) and the α-aminitin resistant activity; the latter was taken as form A activity. Finally, form A and B activities of nuclei from drug-treated cells were expressed as percentage of control values.

RESULTS

Effects of Eth Br on $^3$H-uridine incorporation into total nuclear, cytoplasmic and Hn RNA species.

Mouse kidney cells, either uninfected or infected with SV40, Monkey kidney cells, SVMK clone 9, or CV-1 cells were treated with either 2 or 4 µg/ml of Eth Br for 6 hr, and were incubated with 10 µCi/ml $^3$H-uridine during the last three hours of Eth Br-treatment. The amount of radioactivity in the nuclear and cytoplasmic fractions was determined in cpm/µg DNA as described in Methods and then expressed a percentage of control values (cells not treated with the drug served as controls) (Table 1). In some experiments the contribution of ribosomal RNA to the labeling of nuclear RNA was eliminated by treating cells with 0.05 µg/ml actinomycin D, in order to study the effects of Eth Br on the labeling of heterogeneous nuclear RNA (Hn RNA) (Table 1). The results show that in mouse cells, Eth Br depresses the labeling of total nuclear RNA, and to a greater extent that of cytoplasmic RNA. The labeling of Hn RNA was slightly reduced or was unaffected. Under these experimental conditions, we have found that the contributions of ribosomal RNA and Hn RNA to total nuclear RNA are approximately 65 % and 35 %, respectively (data not shown). Table 1 also shows that confluent mouse cells ($6 \times 10^4$ cells/cm$^2$) appear relatively less sensitive to Eth Br than mouse cells maintained at low cell density ($3 \times 10^4$ cells/cm$^2$). SV40-transformed mouse cells are less sensitive to the drug than untransformed mouse cells and the CV-1 monkey cells appear to be particularly drug resistant. Primary cultures of monkey kidney cells are more sensitive to Eth Br than are CV-1 cells. Moreover, the effect of the drug on confluent BMK cells, whether or not infected with SV40, are comparable, although SV40-infected cells might be slightly more sensitive.

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to the drug than are uninfected cells. It is relevant to indicate that a study parallel to this one was carried out on the effect of Eth Br on the transcription of the SV40 genome in BMK cells (in preparation).

Effects of Eth Br treatment on form A and B RNA polymerase activities estimated in isolated nuclei.

In the above experiment we examined the rates of accumulation for the various nuclear RNA species. The reduction of $^3$H-uridine incorporation into total nuclear and Hn RNA seen in Eth Br-treated cells could reflect a diminished rate of RNA synthesis, an alteration in RNA processing or transport, a reduction in RNA stability or an alteration in nucleotide pool size. In an attempt to distinguish between these possibilities, endogenous activities of A and B DNA-dependent RNA polymerase were estimated in isolated nuclei from confluent BMK cells, either with or without drug treatment, using 1 µg/ml α-aminonucleotide added 30 minutes before $^3$H-uridine.

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**TABLE I**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Eth Br concentrations µg/ml</th>
<th>Nuclear RNA (%)</th>
<th>Cytoplasmic RNA (%)</th>
<th>Heterogeneous nuclear RNA (+) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baby mouse kidney (cultures at low cell density, 5 x 10⁶ cells/cm²)</td>
<td>2</td>
<td>52 ± 7 (3)</td>
<td>28.1 ± 7 (3)</td>
<td>71.8 ± 8.3 (3)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>39.5 ± 7.3 (3)</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Baby mouse kidney (confluent cultures; 6 x 10⁶ cells/cm²)</td>
<td>2</td>
<td>78.3 ± 5.1 (3)</td>
<td>46.2 ± 5.9 (3)</td>
<td>98.6 ± 7.5 (4)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>67 ± 4.7 (3)</td>
<td>37.8 ± 7.4 (3)</td>
<td>N.D.</td>
</tr>
<tr>
<td>SV40-infected confluent baby mouse kidney</td>
<td>2</td>
<td>62.1 ± 5 (2)</td>
<td>57.3 ± 6 (2)</td>
<td>N.D.</td>
</tr>
<tr>
<td>SV40 clone 9</td>
<td>2</td>
<td>97.5 ± 4.4 (2)</td>
<td>99.3 ± 7.5 (2)</td>
<td>103 ± 5 (2)</td>
</tr>
<tr>
<td>Monkey kidney cells (confluent primary cultures)</td>
<td>4</td>
<td>81.4 ± 0.8 (2)</td>
<td>51.8 (1)</td>
<td>N.D.</td>
</tr>
<tr>
<td>CV-1</td>
<td>2</td>
<td>97 ± 4.2 (2)</td>
<td>102 ± 4 (2)</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>90.3 ± 0.8 (2)</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

The cells were labeled during the last 3 hr of a 6-hr drug treatment with 10 µCi/ml $^3$H-uridine in the presence of 5 µg/ml cold uridine. For control cells, the Eth Br was omitted. The amount of radioactivity incorporated into the total nuclear, cytoplasmic and heterogeneous nuclear RNAs were determined in cpm/µg DNA and expressed as percentages of the control values. Each figure represents the mean ± SD. Number in parentheses indicates the number of experiments.

(+) The contribution of ribosomal RNA to the labeling of nuclear RNA was suppressed by treating the cells with 0.05 µg/ml actinomycin added 30 minutes before $^3$H-uridine.

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view that Eth Br acts to decrease the rate of transcription of nuclear genes, especially ribosomal RNA genes.

Cumulative and reversible effects of Eth Br on nuclear transcription.

In order to examine whether or not the effect of Eth Br on nuclear RNA-synthesizing machinery was cumulative, one set of BMK cells at low cell density (3 x 10^4 cells/cm^2) was treated with 4 μg/ml Eth Br for various time spans ranging between 1.5 and 6 hr, and was labeled with 10 μCi 3H-uridine during the last hour of drug treatment. In the same experiment another set of BMK cells at low cell density (3 x 10^4 cells/cm^2) was treated with Eth Br (4 μg/ml) for 6 hr and then washed and re-covered with fresh medium without Eth Br (and without serum). At different time intervals after the maintenance medium containing Eth Br had been replaced by Eth Br-free medium, the cells were labeled for 3 hr with 10 μCi/ml 3H-uridine. After the labeling, the cells were processed as above to determine the amount of radioactivity incorporated into the nuclear fraction in cpm/μg DNA, and this was expressed as a percentage of control values. Figure 1 shows that the inhibitory effect of the drug treatment on nuclear RNA synthesis becomes more pronounced with increasing exposure time and that nuclear RNA synthesis declines to about 35% of the value in the control cells after a 6-hr treatment. After removal of the drug, synthesis returned to 93% of control within 9 hr and to normal within 15 hr. These results suggest that the nuclear (especially the nucleolar) RNA synthesizing ma-
Fig. 1: Cumulative and reversible effect of Eth Br treatment

Panel a - BMK cells at a low density (3 x 10^4 cells/cm^2) were treated with 4 μg/ml Eth Br for the period shown on the abscissa. They were labeled with 10 μCi/ml ^3H-uridine for the last hour of treatment. For control cells, the Eth Br was omitted.

Panel b - BMK cells at a low density (3 x 10^4 cells/cm^2) were treated for 6 hr with 4 μg/ml Eth Br. At various time intervals after the maintenance medium had been replaced by Eth Br-free medium, the cells were labeled with 10 μCi/ml ^3H-uridine for a 3-hr period ending at the time indicated on the abscissa. For control cells, the Eth Br was omitted.

Panels a and b - After the labeling, the cells were processed as described in text to determine the amount of radioactivity incorporated into the nuclear fraction, in cpm/μg DNA. This was then expressed as a percentage of control values (ordinate) (From the time of initiation of drug-treatment, the culture medium was serum-free).

The nuclear transcription machinery is progressively affected by the Eth Br treatment but does not undergo irreversible damage since a total recovery of RNA synthesis occurs within 15 hours of removal of the drug.

Comparison of the effects of Eth Br treatment with those of a chloramphenicol treatment.

The effect of Eth Br on the nuclear transcription machinery might be a consequence of the inhibition of mitochondrial protein synthesis caused by the drug. To test this hypothesis, we compared the effects of a 6-hr treatment with Eth Br (4 μg/ml) with those of a 6-hr treatment with chloramphenicol (50 μg/ml) on confluent BMK cells and on BMK cells maintained at low cell density (3 x 10^4 cells/cm^2). The data (Table III) show that the
effects of Eth Br vary as a function of cell density whereas those of chloramphenicol are less pronounced and are independent of cell density. These results suggest that the decrease in nuclear transcription caused by Eth Br is not simply due to the suppression of mitochondrial protein synthesis.

**Effect of Eth Br on rRNA maturation.**

Eth Br was next studied for its effect on the labeling of nuclear and cytoplasmic RNA subspecies of mouse cells. SV40-infected confluent BMK cell cultures were treated with 2 μg/ml Eth Br for either 6 or 6.5 hr, and were incubated with 10 μCi/ml 3H-uridine for either the last 3 hr or the last 6 hr of treatment respectively. These cultures were compared to non Eth Br-treated controls. The electrophoretograms of nuclear RNA from drug-treated and control cells (Fig. 2) show the occurrence of the three main peaks corresponding to 45 S, 32 S and 28 S RNA species. Clearly the electrophoretogram (Fig. 2) reveals no abnormal accumulation of 28 S RNA species in the nuclei of drug-treated cells relative to the control cells. The electrophoretograms of cytoplasmic RNA (Fig. 2) as well as the sedimentation patterns (Fig. 3) of cytoplasmic Poly(A)-RNA from the same cells indicate that the drug reduces the incorporation of 3H-uridine into the 28 S rRNA, 18 S rRNA and the labeling of 28 S rRNA is inhibited to a greater extent than is that of 18 S rRNA. The amounts of radioactive 28 S, 18 S and 7-4 S RNA species were determined after a 3-hr labeling by resolving graphically the sedimentation patterns of cytoplasmic Poly(A)-RNA into the three separate peaks. These amounts expressed as a percentage of the corresponding
control values are represented in Table IV. The percentage of cpm in the Poly(A)+ RNA from the treated cells as compared to the control values is also given in Table IV. These results are corrected for cytoplasmic RNA content. In the cytoplasm, the drug inhibits the labeling of the various types of RNA in the decreasing order: 28 S rRNA > 18 S rRNA > 7-4 S RNA > Poly(A)+ mRNA. Since there is no abnormal accumulation of 28 S rRNA in the nuclei of drug-treated cells, the fact that the ratio of 28 S : 18 S radioactive cytoplasmic RNA is diminished relative to that of control cultures is probably due not to a slowdown of 28 S nucleocytoplasmic transfer, but to a decreased stability of 28 S rRNA or a slowdown in the processing of 32 S to 28 S rRNA. This interpretation is consistent with the fact that the decline in the ratio of 28 S : 18 S radioactive cytoplasmic RNA is practically the same after either a 3-hr or 6-hr labeling period (Figure 3).

DISCUSSION

From our results it is clear that relatively low concentrations (2 or 4 μg/ml) of Eth Br inhibit markedly the labeling of various RNA species originating in the nucleus of cultured mouse cells. These concentrations are close to those (≤ 1 μg/ml) for which the drug is considered as a selective inhibitor of mitochondria-associated RNA (12, 13, 14).

The drug appears to exert its inhibition at various stages of nuclear-coded RNA metabolism. First, an inhibitory effect is exerted upon the transcription of nuclear genes, especially of ribosomal RNA genes, as

Fig. 2: Polyacrylamide gel electrophoresis of nuclear and cytoplasmic RNA from SV40-infected BMK cells labeled with 3H-uridine.

SV40-infected BMK cells were exposed to 2 μg/ml Eth Br from 3 to 9 hours p.i. For control cells, the Eth Br was omitted. They were labeled with 10 μCi/ml 3H-uridine from 6 to 9 hours p.i. At the end of the labeling period, cells were fractionated to obtain nuclear and cytoplasmic fractions (cf Methods). The RNA from the nuclear fraction was extracted with hot phenol-SDS; the RNA from the cytoplasmic fraction was extracted by the phenol-chloroform method. The RNA populations were analyzed by electrophoresis on 2.2 % polyacrylamide gels.

Gels were 0.6 cm in diameter and 13 cm in length, and had a 1.5 cm 5 % acrylamide cushion at the bottom. Electrophoresis was carried out at 10 V/cm and 10°C for 270 min (nuclei) or for 105 min (cytoplasm). Gels were cut into 2 mm slices and each slice was incubated with 0.5 ml NCS (Amersham) for 2 hr at 50°C before counting in 10 ml of scintillator.

Upper panels: nuclear RNA
Lower panels: cytoplasmic RNA
Effects of Eth Br on the sedimentation patterns of cytoplasmic Poly(A)-RNA.

SV40-infected mouse kidney cells were treated with 2 μg/ml Eth Br for 6 hr (a and b) or 6.5 hr (c and d) and labeled with 10 μCi/ml 3H uridine for the last 3 hr (a and b) or 6 hr (c and d) of the drug treatment. For control cells, the Eth Br was omitted. The cytoplasm was recovered from the cells by treatment of washed monolayers with isotonic buffer containing 1% Nonidet P 40. The cytoplasmic RNA was separated into poly(A)+ RNA and poly(A)-RNA by chromatography on oligo dT-cellulose. Poly(A)-RNA was analyzed by sedimentation in a 15-30% (w/v) sucrose gradient (10 mM triethanolamine pH 7.4, 50 mM NaCl, 1 mM EDTA). Sedimentation was carried out at 54 K rpm in a Spinco SW 56 rotor for 110 min at 20°C.

Panels a and c: control; Panels b and d: drug-treated.
judged by the decline in form A and, to a lesser extent, form B RNA polymerase activity as estimated in isolated nuclei after drug-treatment. These decreases are comparable to, and might account for, those observed in the labeling of total nuclear RNA (mainly ribosomal RNA) and Hn RNA species respectively.

This effect becomes more pronounced with exposure time; however, after removal of the drug, there is a progressive recovery of RNA synthesis culminating in the complete reversal of the drug effect. These observations suggest that under our experimental conditions, Eth Br-treatment causes a progressive but reversible alteration of the nuclear (especially nucleolar) RNA synthesizing machinery. It is worth noting that Simard(29), studying the effects of Eth Br on cultured rat cells at the ultrastructural level has observed that a nucleolar segregation was the only lesion caused by the drug at a low dose (2 μg/ml for 6 hours). Our results appear to be consistent with Simard's observation, although we must bear in mind that the sensitivity of the cells to Eth Br depends on the animal species.

This alteration of nuclear RNA synthesizing machinery is probably not simply due to the suppression of mitochondrial protein synthesis since (i) a treatment with 50 μg/ml of chloramphenicol (a specific and complete inhibitor of mitochondrial protein synthesis (17)) affects the nuclear RNA labeling significantly less than does Eth Br-exposure, and (ii), a preferential effect of the drug on BMK cell cultures at low cell density relative to confluent BMK cell cultures, when compared on a nuclear RNA labeling basis, is observed with Eth Br but not with chloramphenicol.

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**TABLE IV**

EFFECT OF ETH BR ON THE LABELING OF VARIOUS CYTOPLASMIC RNA SPECIES.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Eth Br concentration, μg/ml</th>
<th>28 S RNA</th>
<th>18 S RNA</th>
<th>4-7 S RNA</th>
<th>Poly(A)+ RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV40-infected mouse</td>
<td>2</td>
<td>21.6 ± 1.1 (2)</td>
<td>37 ± 3.5 (2)</td>
<td>42.5 ± 0.1 (2)</td>
<td>76 ± 6.4 (4)</td>
</tr>
<tr>
<td>kidney</td>
<td>4</td>
<td>10.4 ± 2.6 (2)</td>
<td>19.8 ± 3.5 (2)</td>
<td>27.8 ± 2.3 (2)</td>
<td>34.9 ± 4.8 (4)</td>
</tr>
<tr>
<td>SV40 Clone 9</td>
<td>2</td>
<td>26 ± 6.9 (2)</td>
<td>37.6 ± 9.8 (2)</td>
<td>84.5 ± 3.5 (2)</td>
<td>106 ± 14.1 (2)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.6 ± 0.8 (2)</td>
<td>10.2 ± 4.8 (2)</td>
<td>35.8 ± 3.5 (2)</td>
<td>92.8 ± 3.6 (2)</td>
</tr>
</tbody>
</table>

The cells were exposed to Eth Br (2 or 4 μg/ml) for 6 hr and labeled with 10 μCi/ml 3H-uridine in the presence of 5 μg/ml cold uridine during the last 3 hr of drug treatment. For control cells the Eth Br was omitted. The cytoplasmic fraction was prepared by disrupting the cells in a buffered 2% solution of Nonidet P-40. Cytoplasmic RNA was extracted by the phenol-chloroform method and separated into poly(A)+ and poly(A)- RNA by chromatography on oligo(dT)-cellulose. Poly(A)- RNA was then fractionated and separated into 28 S RNA, 18 S RNA and 4-7 S RNA by sedimentation on sucrose gradient.

The amount of radioactivity associated with each fraction was corrected for RNA content. The results are expressed in percentage of the control values. Each figure represents the mean ± SD. Number in parentheses indicates number of experiments.
There is, in addition, an effect of Eth Br on rRNA maturation. Eth Br depresses the labeling of cytoplasmic rRNA more than that of nuclear RNA. In the cytoplasm, it depresses the labeling of 28 S rRNA more than that of 18 S rRNA; this has been observed after a 6-hr labeling as well as after a 3-hr labeling. Since there is apparently no abnormal accumulation of 28 S RNA in the nuclei of drug-treated cells (Figure 2), it is suggested that the drug treatment may affect the stability of the 28 S rRNA or its rate of formation from the 32 S precursor. Since it has been shown that proteins are released from ribosomes by Eth Br (30) through intercalation of the drug in the double-stranded rRNA regions (6), the effect of Eth Br on rRNA maturation may reside at the level of conversion of pre-rRNA into stable ribosomal particles. The possibility that Eth Br or proflavine may affect the ribosomal RNA maturation processing was already suggested by Snyder et al. (18) and Yannarell et al. (31), but these authors employed very high concentrations of the drug.

In conclusion, the present results indicate that the biological consequences of treating cultured mouse cells with Eth Br may be multiple, even with drug concentrations as low as 5 μM - 10 μM.

ACKNOWLEDGEMENTS

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